

Calcineurin Is Required for *Candida albicans* To Survive Calcium Stress in Serum

Jill R. Blankenship¹ and Joseph Heitman^{1,2,3,4*}

Departments of Molecular Genetics and Microbiology,¹ Medicine,² and Pharmacology and Cancer Biology,³ and Howard Hughes Medical Institute,⁴ Duke University Medical Center, Durham, North Carolina 27710

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The calcium-activated protein phosphatase calcineurin plays a critical role in the virulence of *Candida albicans*. Previous studies demonstrated that calcineurin is not required for the yeast-hypha dimorphic transition, host cell adherence, or host cell injury, which are all established virulence attributes of this organism. Calcineurin is, however, essential for survival in serum and disseminated infection. Here we identify the component of serum that is toxic to calcineurin mutant cells. Proteins, peptides, lipids, and other hydrophobic components were all excluded as essential toxic elements. Upon testing of small molecules present in serum, we discovered that calcineurin protects cells from stress caused by the endogenous levels of calcium ions present in serum. These studies illustrate how calcineurin functions in a calcium homeostatic pathway that enables a common human commensal to survive passage through the hostile environment of the bloodstream to establish deep-seated infections and cause disease.

Calcineurin is a calcium-activated serine-threonine-specific protein phosphatase that is conserved from yeasts to mammals. This phosphatase is composed of a regulatory (B) and a catalytic (A) subunit that form an inactive AB heterodimeric complex; calcium-induced binding by calmodulin elicits conformation changes leading to enzyme activation (1, 23, 28, 43). An influx of calcium ions into the cytoplasm of eukaryotic cells from either the extracellular environment or intracellular stores leads to calmodulin and calcineurin activation (41). In fungal cells, this influx of Ca^{2+} is often triggered by diverse environmental stresses, such as temperature fluctuation, high extracellular ionic concentrations, and changes in extracellular pH. In *Saccharomyces cerevisiae*, calcineurin activation leads to downstream events that mediate cellular responses to stress and concomitantly reduce the concentration of Ca^{2+} within the cytoplasm to basal levels (14).

Recent studies reveal that calcineurin is essential for the virulence of two divergent pathogenic fungi, *Cryptococcus neoformans* and *Candida albicans* (2, 7, 38, 44). Although the phenotypes conferred by calcineurin inhibition or mutation share common features in the two fungi, the roles that this phosphatase fulfills in virulence differ. Calcineurin is essential for *C. neoformans* survival at mammalian physiological body temperatures (37°C and higher) (12, 38), and as a consequence, calcineurin mutants of this pathogen fail to produce lethal infections in animal models of pathogenesis. Calcineurin is not, however, required for the survival of *C. albicans* at elevated temperatures (7). Instead, *C. albicans* calcineurin is essential for survival in serum, and as a consequence, mutants lacking the calcineurin A or B subunit are compromised for survival in vivo.

Serum is the liquid component of blood that remains after clotting has occurred and fibrous, insoluble material has been removed. Serum is an undefined, complex medium composed of proteins, lipids, and small molecules, and the interaction of *C. albicans* with serum has been of long-standing interest in the fungal pathogenesis field. Early work suggested that serum had a lethal effect on *C. albicans* and that this activity was heat stable and dialyzable (30), but it was later demonstrated that the reduced numbers of CFU measured by these experiments were attributable to increased aggregation of *Candida* cells rather than to a lethal effect of serum (10). This serum-induced aggregation is related to serum stimulation of the yeast-to-hypha transition, and though the search for the switching stimulus in serum has been one of the most enduring questions in *Candida* pathogenesis (4, 17, 25, 36, 46), no obvious culprit has been definitively identified yet. Recent studies reveal that serum does become profoundly toxic to *C. albicans* when calcineurin is either inhibited or mutated (7, 44), findings reminiscent of the early *C. albicans*-serum work. The requirement of calcineurin for *C. albicans* survival in serum sparked our interest in searching for serum components that are toxic to calcineurin mutant cells. Here we describe experiments that reveal the component of serum toxic to *C. albicans* calcineurin mutants.

MATERIALS AND METHODS

Strains and media. The *C. albicans* calcineurin mutant strain JRB64 (*ura3Δ::nim43Δ/ura3Δ::nim43Δ his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG cnb1::UAU1/cnb1::ARG4*) (11), the complemented calcineurin mutant strain MCC85 (*ura3Δ::nim43Δ/ura3Δ::nim43Δ his1::hisG::CNB1-HIS1/his1::hisG arg4::hisG/arg4::hisG cnb1::UAU1/cnb1::ARG4*) (11), *crz1* mutant strains OCC1.1 and OCC3.8 (*ura3Δ::nim43Δ/ura3Δ::nim43Δ his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG crz1::UAU1/crz1::ARG4*) (40), *crz2* mutant strains DAY686 and DAY697 (*ura3Δ::nim43Δ/ura3Δ::nim43Δ his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG crz2::UAU1/crz2::ARG4*) (generous gifts from Dana Davis prior to publication), strain DAY185, marker-matched to the mutant and complemented strains (*URA3/ura3Δ::nim43Δ HIS1/his1::hisG ARG4/arg4::hisG*) (15), and the wild-type reference strain SC5314 (18) were used in this study. Strains were grown in yeast extract-peptone-dextrose (YPD) rich medium pre-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, 322 CARL Building, Box 3546, Duke University Medical Center, Research Drive, Durham, NC 27710. Phone: (919) 684-2824. Fax: (919) 684-5458. E-mail: heitman001@duke.edu.

pared as previously described (45), fetal bovine serum (FBS), murine serum, porcine serum, or ovine serum (all sera were from Sigma). Fresh human serum collected from a healthy volunteer was also used as a growth medium for *C. albicans* strains. YPD and FBS media were supplemented with FeCl_3 , ZnSO_4 , CaCl_2 , 1,2-bis(2-aminophenoxy)-ethane- N,N,N',N' -tetraacetic acid (BAPTA), and/or EGTA (all chemicals were from Sigma) at the concentrations indicated in the text.

Liquid growth assays. For all growth experiments, wild-type, calcineurin mutant, or complemented mutant cells were grown overnight at 30°C in liquid YPD medium. Cells were pelleted, washed once in phosphate-buffered saline (PBS), and then counted with a hemocytometer. A total of 5×10^3 cells/ml of each strain were incubated in the indicated media and rotated on a roller drum or incubated in a 96-well culture dish (Corning Inc., NY) at 30°C for the times specified. Aliquots of the cultures were removed at the initial time point (time zero) as well as at the termination of the experiment, and CFU were determined following appropriate dilutions of samples. Fold population change was measured by dividing the CFU at the experimental time point by the CFU measured at the initial time point. Values above 1 indicate growth of the culture, values near 1 indicate culture stasis, and values below 1 indicate cell loss or cell death.

Probing serum proteins. FBS was filtered through Centricon filters (Millipore) with 30-kDa, 10-kDa, and finally 3-kDa molecular mass exclusion sizes according to the manufacturer's instructions. The filtrates and retentates were tested for activity against the calcineurin mutant strain in a liquid growth assay following reconstitution of the samples to the original volume with sterilized, distilled water to achieve the original concentrations. The wild-type, calcineurin mutant, and complemented calcineurin mutant strains were tested in the liquid growth assay using boiled, clarified FBS to further assess serum protein activity against calcineurin mutant strains. FBS was heated to boiling temperature for 10 min in a double-boiler system to prevent the serum from scorching. Any insoluble denatured proteins were removed by centrifugation at 3,000 rpm in a Sorvall RT 6000D centrifuge. Following centrifugation, sterile distilled water was added to adjust the solution to the original concentration, and this boiled, clarified serum was tested for activity against a *C. albicans* calcineurin mutant strain. Finally, serum that had been incubated with 50 $\mu\text{g}/\text{ml}$ proteinase K for 16 h at 30°C to digest serum proteins and peptides was tested for activity against the reference, calcineurin mutant, and complemented calcineurin mutant strains. Similarly treated YPD was used to control for any influence the active proteinase K might have on strain growth. Untreated serum and YPD were also incubated at 30°C for 16 h and used for comparison.

Lipids in serum. To determine if lipids or other hydrophobic serum components contribute to lethal activity against calcineurin mutant strains of *C. albicans*, the aqueous layer of chloroform-extracted FBS was tested for toxicity. An equal volume of chloroform was added to FBS and vortexed. The FBS-chloroform solution was centrifuged for 15 min at 3,000 rpm in a Sorvall RT 6000D centrifuge, and the aqueous layer was removed and lyophilized. The dried aqueous phase of serum was reconstituted with water and then tested for activity against calcineurin mutant strains.

Ca^{2+} concentration in serum. The concentration of calcium ions in FBS and YPD was measured by using a calcium colorimetric assay from Point Scientific (Lincoln Park, MI) according to the manufacturer's guidelines. In the absence of Ca^{2+} , the detection solution is yellow, whereas the presence of Ca^{2+} yields a purple color. The color intensity is proportional to the sample Ca^{2+} concentration. A 20- μl volume of each medium was added to the reagents supplied in the kit, and the optical density of these solutions at a wavelength of 570 nm was compared to a Ca^{2+} standard by using a Beckman DU 640 spectrophotometer.

RESULTS

Serum is lethal to *C. albicans* in the presence of calcineurin inhibitors. It was previously demonstrated that serum is lethal to strains lacking calcineurin (7). To establish whether this serum lethality is observed in wild-type cells by the addition of a calcineurin inhibitor, we tested the effect of supplementing serum with FK506. The reference (SC5314), calcineurin mutant (JRB64) (*cnb1/cnb1*), and calcineurin-reconstituted (*cnb1/cnb1+CNB1*) strains, as well as a strain marker-matched to the calcineurin mutant and reconstituted strains (DAY185) (15), were added to serum containing increasing concentrations of FK506. The calcineurin mutants were unable to survive in serum regardless of FK506 addition, and the reference, mark-

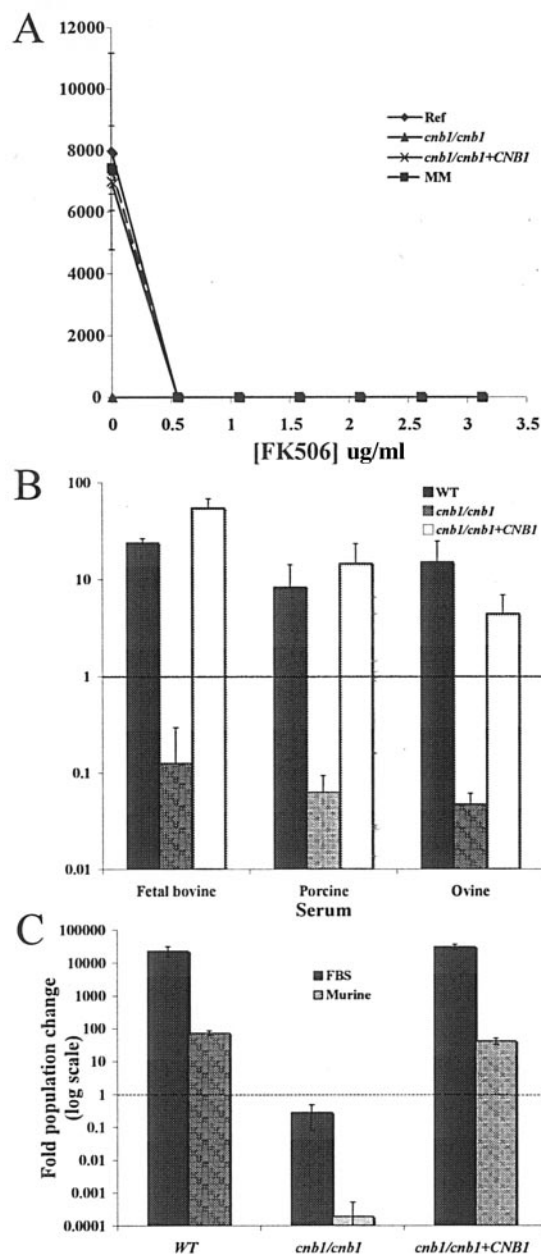


FIG. 1. Serum is lethal to wild-type cells in the presence of calcineurin inhibitors, and toxicity is not unique to fetal bovine serum. (A) The reference strain (SC5314) (\blacklozenge), a marker-matched strain (DAY185) (\blacksquare), a *cnb1/cnb1* calcineurin B mutant strain (JRB64) (\blacktriangle), and a calcineurin B-complemented strain (MCC85) (\times) were grown overnight in fetal bovine serum supplemented with FK506 at the indicated concentrations. (B and C) The reference strain (SC5314) (WT), the *cnb1/cnb1* calcineurin B mutant strain (JRB64), and the *cnb1/cnb1+CNB1* calcineurin B-complemented strain (MCC85) were tested for survival in 100% fetal bovine, porcine, ovine (B), or murine (C) sera. Approximately 5,000 cells/ml of each strain were incubated in the indicated media for 9 h (B) or 48 h (C) at 30°C.

er-matched, and reconstituted strains exhibited partial sensitivity to serum in the presence of 0.04 $\mu\text{g}/\text{ml}$ FK506 and complete serum sensitivity in the presence of 0.55 $\mu\text{g}/\text{ml}$ FK506 (Fig. 1A and data not shown). We note that in this experiment,

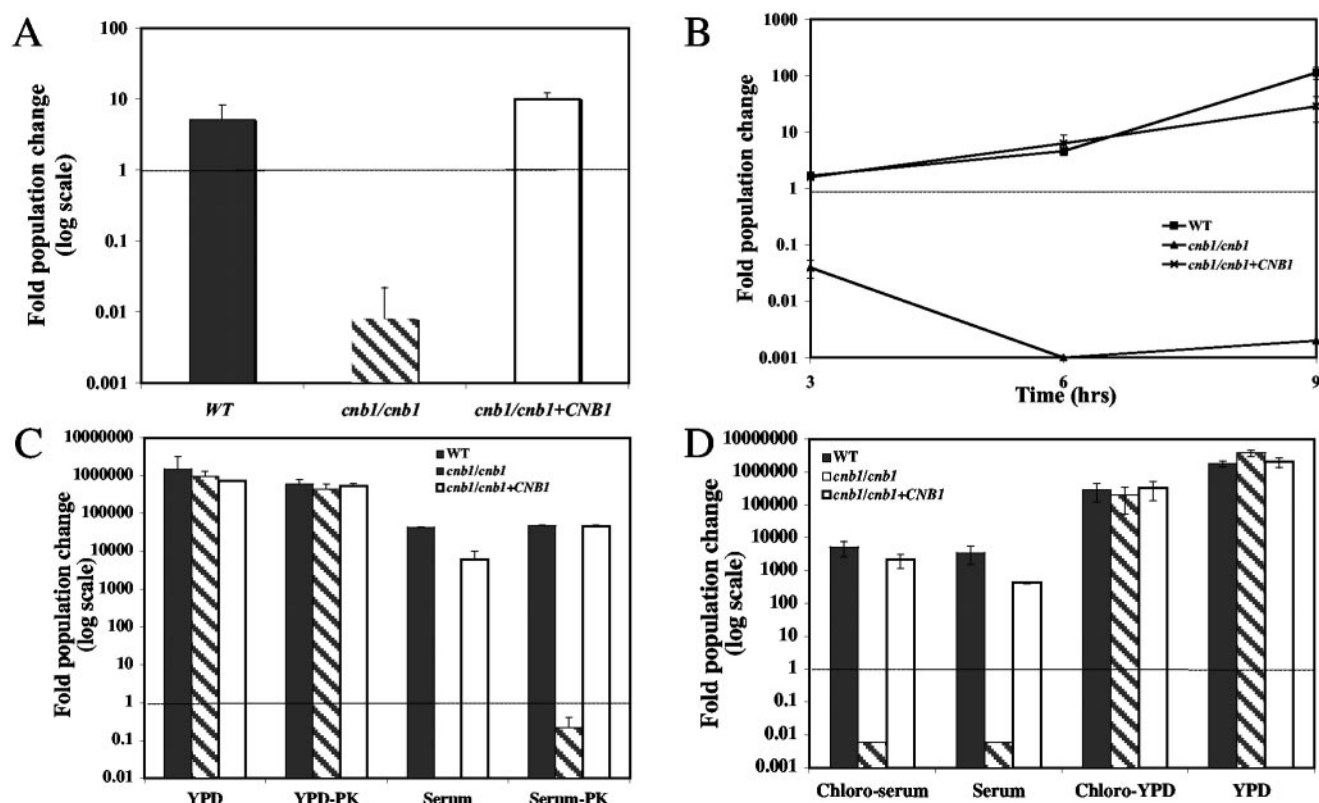


FIG. 2. The lethal factor of serum is not a protein or a lipid. (A) The wild-type strain (SC5314) (solid bar), a *cnb1/cnb1* calcineurin B mutant (JRB64) (shaded bar), and a *cnb1/cnb1+CNB1* calcineurin B-complemented strain (MCC85) (open bar) were tested for survival at 9 hours in fetal bovine serum that had been filtered through a 3-kDa Centricon filter. (B) The survival of wild-type (■), *cnb1/cnb1* (▲), and *cnb1/cnb1+CNB1* (×) strains was measured at the indicated times in the soluble fraction of fetal bovine serum that had been boiled and centrifuged to denature and remove proteins. (C) The survival of wild-type (solid bars), *cnb1/cnb1* (shaded bars), and *cnb1/cnb1+CNB1* (open bars) strains in YPD, proteinase K-pretreated YPD (YPD-PK), serum, or proteinase K-pretreated serum (serum-PK) was measured at 24 h. (D) Survival of wild-type (solid bars), *cnb1/cnb1* (shaded bars), and *cnb1/cnb1+CNB1* (open bars) strains was measured at 24 h in chloroform-extracted serum, serum, chloroform-extracted YPD, or YPD that had been lyophilized and reconstituted.

as well as in several other experiments, DAY185 and SC5314 exhibited nearly identical phenotypes. To simplify results, SC5314 was used as the reference strain in the remainder of the studies presented here and will henceforth be referred to as the wild-type strain.

Serum from diverse sources is toxic to *C. albicans* calcineurin mutants. Fetal bovine serum supports the survival and growth of wild-type *C. albicans* cells but not of calcineurin mutant cells (7, 44). We sought to identify the factor(s) in serum that is toxic to *C. albicans* calcineurin mutants in order to provide insight into the role of calcineurin in virulence. However, serum is a complex and undefined medium, complicating this analysis. To rule out the possibility that the killing activity of serum is attributable to a species-specific factor, porcine, ovine (sheep), murine, and human sera were also tested for their toxicity against calcineurin mutant cells. The growth of wild-type cells in porcine and ovine sera was similar to that in FBS, and calcineurin mutant cells were unable to survive in the presence of 100% serum from any of these sources (Fig. 1B). Growth of wild-type *C. albicans* cells was much slower in murine serum (~6 population doublings in murine serum versus ~14.5 population doublings in FBS at 48 h) and fresh human serum (only ~4.2 population doublings

after 24 h), and calcineurin mutants also failed to survive in these media, as in FBS (Fig. 1C and data not shown). We conclude that the factor toxic to *C. albicans* calcineurin mutant cells is a ubiquitous component of sera.

The lethal factor in serum is not proteinaceous. A series of experiments was conducted to determine whether the lethal factor in serum is a protein, peptide, lipid, or small molecule. Serum contains approximately 60 to 80 mg/ml protein, and we first tested whether one or more of these proteins might be toxic to *C. albicans* strains lacking calcineurin. Because separating and testing individual serum proteins would be laborious, and more than one protein could contribute to lethality, we first tested whether the killing activity was retained in serum in which proteins had been denatured and removed, and whether activity would be retained in serum subjected to proteinase K treatment.

Serum fractions of >30 kDa, 10 to 30 kDa, 3 to 10 kDa, and <3 kDa were obtained by sequential filtration and tested for killing activity against calcineurin mutant cells. The <3-kDa fraction retained killing activity against calcineurin mutant strains, indicating that if the toxic activity is attributable to protein, it would have to be relatively small (<30 amino acids) (Fig. 2A). Serum fractions of >3 kDa did not support growth

of the calcineurin mutant strains either, but the toxicity of combined fractions of >3 kDa is much reduced (a fold population change of ~ 0.6 at 9 h, more than 73-fold higher than that in the <3 -kDa fraction). This moderate toxicity might be due to intrinsic activity or to a small molecule (<3 kDa) bound to one or more proteins in the >3 -kDa fraction of serum. Next, serum proteins were denatured by boiling and removed by centrifugation, and the supernatant of the boiled, clarified serum was tested for activity against calcineurin mutant strains. This boiled serum retained toxicity against calcineurin mutant cells, providing evidence that the toxic serum factor is not proteinaceous (Fig. 2B). One caveat is that while most proteins are destroyed by this treatment, small peptides (including antimicrobial peptides) might both pass through a 3-kDa Centricon filter and survive boiling.

To further eliminate proteins as a potential lethal factor, *C. albicans* cells were incubated in serum pretreated with 50 $\mu\text{g/ml}$ (1.5 U/ml) proteinase K for 16 h at 30°C . Calcineurin mutant strains were still killed by the protease-treated serum, whereas the growth of wild-type cells was unaffected (Fig. 2C). Proteinase K was not inactivated in this experiment, and to determine whether the protease activity itself influenced calcineurin mutant survival, wild-type, calcineurin mutant, and complemented calcineurin mutant strains were tested for survival in proteinase K-treated YPD. Proteinase K treatment of YPD did not affect the growth of the calcineurin mutant or wild-type strains of *C. albicans* (Fig. 2C). We conclude that the toxic component of serum is not a protein or a peptide.

The lethal factor of serum is not a lipid. Calcineurin is essential when the membrane of *C. albicans* is stressed, such as following inhibition of ergosterol biosynthesis inhibitors or after treatment with sodium dodecyl sulfate (11). The incorporation of mammalian lipids or cholesterol from serum into the membrane of *C. albicans* might evoke a similar stress, requiring calcineurin signaling for survival. Lipids and other hydrophobic molecules typically found in the cell membrane, such as sterols, are abundant in serum (4 to 8 mg/ml). Incorporation of cholesterol (the mammalian equivalent of the fungal sterol ergosterol) by a related species, *Candida glabrata*, has been observed (35), and thus uptake of sterols and lipids by *C. albicans* might occur in the lipid-rich environment of serum.

To determine whether lipids or other hydrophobic molecules contribute to serum lethality, serum and YPD were chloroform extracted, and the aqueous supernatant was tested for activity against calcineurin mutant and wild-type cells. The presence of even trace amounts of chloroform was lethal to *C. albicans* cells (data not shown), and so the hydrophobic phase of the extraction could not be tested for toxicity against calcineurin mutant cells. The aqueous phase of the chloroform extraction was lyophilized and reconstituted with sterile distilled water prior to testing. Assays were performed with both extracted YPD and serum as well as unextracted media to control for any effects that lyophilization might have on survival. Chloroform extraction and lyophilization had no effect on calcineurin-dependent survival of the *C. albicans* strains tested, and chloroform-extracted serum retained activity against calcineurin mutant cells at a level comparable to that of native serum (Fig. 2D). Furthermore, the addition of 0.25 M cholesterol to PBS (~ 12 to 24 times the amount present in serum) did not affect the survival of calcineurin mutant or

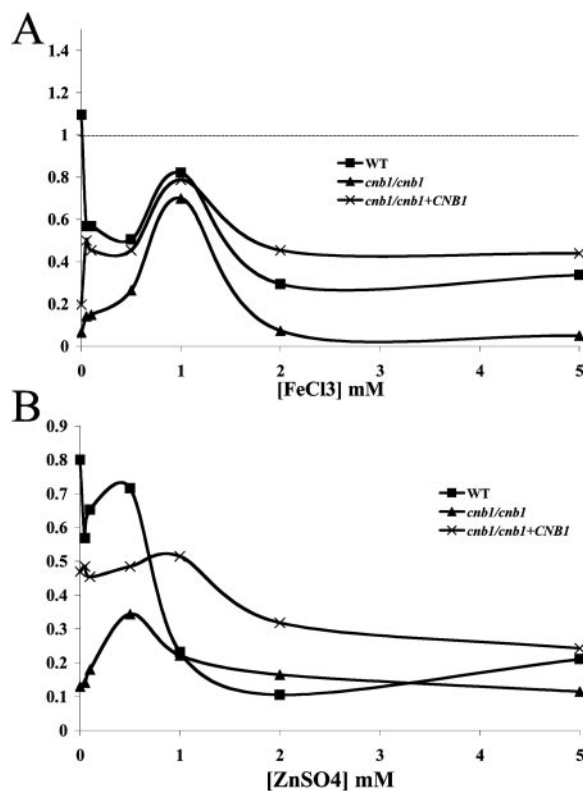


FIG. 3. Calcineurin does not mediate responses to iron or zinc toxicity. Wild-type (SC5314) (■), *cnb1/cnb1* (JRB64) (▲), and *cnb1/cnb1+CNB1* (MCC85) (×) strains were tested for their abilities to persist in PBS with increasing concentrations of FeCl_3 (A) or ZnSO_4 (B). Cells were incubated for 24 h at 30°C .

wild-type cells (data not shown). We conclude that lipids, sterols, and other hydrophobic molecules present in serum are not the components toxic to cells lacking calcineurin activity.

Fe^{3+} and Zn^{2+} have no calcineurin-dependent antifungal activity. Although these studies exclude proteins, peptides, and lipids as toxic factors, myriad hydrophilic small-molecule components of serum remain that could affect the survival of *C. albicans* calcineurin mutant cells. These include vitamins (which were discussed in a previous publication [7]) and ions, including the metal ions iron and zinc. Iron is essential for life, and host chelation of iron in blood has driven microorganisms to develop methods to scavenge this essential cofactor from chelating proteins and peptides (for a review, see reference 21). However, high levels of iron can be lethal to cells via reactions with oxygen that produce toxic free radicals (20, 48). FeCl_3 was tested for any calcineurin-dependent lethal role it might play in serum toxicity. The addition of 1 mM Fe^{3+} to PBS enhanced the survival of calcineurin mutants over that in PBS alone, but at higher concentrations of Fe^{3+} (2 to 5 mM) this survival benefit was lost, and survival rates returned to levels equivalent to those in PBS alone (Fig. 3A). The survival of wild-type and complemented strains was reduced by the addition of FeCl_3 to PBS from survival in PBS without FeCl_3 (Fig. 3A). Like iron, zinc is also required at low concentrations for cell survival but can be toxic at higher levels. This antimicrobial activity is used clinically to treat topical fungal infec-

tions (16). Also as with iron, the addition of 1 mM ZnSO_4 enhanced the survival of calcineurin mutants in PBS, but 2 to 5 mM ZnSO_4 did not exhibit calcineurin-dependent toxicity (Fig. 3B). Thus, these essential but potentially toxic metals do not appear to play a role in the toxicity of serum against *C. albicans* cells lacking calcineurin function.

Calcium is the toxic component in serum. In previous studies investigating the role of the calcineurin A catalytic subunit in *C. albicans* virulence, calcineurin mutant cells were reported to be hypersensitive to salt stress compared to wild-type controls (2). This included hypersensitivity to 1 mM NaCl, 300 mM LiCl, and 10 mM MnSO_4 , phenotypes shared with *S. cerevisiae* and *C. neoformans*, but it also included hypersensitivity to 300 mM CaCl_2 , a phenotype not shared with these fungi (34, 38). In fact, calcineurin mutant strains of *S. cerevisiae* display increased tolerance to high concentrations of calcium (13). Based on this observation, we investigated whether Ca^{2+} levels in serum might contribute to serum toxicity against calcineurin mutant cells of *C. albicans*.

Prior to testing of the toxicity of Ca^{2+} in serum, the concentrations of Ca^{2+} in FBS and YPD medium were measured. The concentration of Ca^{2+} in 100% FBS ranged from 3.5 to 4 mM, more than 30-fold greater than the concentration of Ca^{2+} in unsupplemented YPD medium (data not shown) but 100-fold less than the concentration tested in the study of Bader et al. (2). Indeed, the addition of 5 mM Ca^{2+} to YPD did not affect the growth of the calcineurin mutant strain (data not shown). However, PBS supplemented with 5 mM CaCl_2 was unable to support the survival of calcineurin mutant strains, whereas both wild-type and complemented calcineurin mutant strains survived (Fig. 4A). Moreover, PBS supplemented with 5 mM CaCl_2 and 1.07 $\mu\text{g/ml}$ FK506 did not support the survival of the wild-type strain (data not shown). To further explore these findings, serum Ca^{2+} was chelated by the addition of the extracellular Ca^{2+} chelator BAPTA. If serum Ca^{2+} levels are toxic to *C. albicans* calcineurin mutant cells, removal of Ca^{2+} should restore growth to this strain. The addition of BAPTA (~ 0.8 mM) to serum rescued the growth of calcineurin mutant cells (Fig. 4B). BAPTA chelates 1 molecule of calcium per BAPTA molecule. Thus, the concentration of BAPTA necessary to rescue the growth of calcineurin mutant cells in serum is ~ 4 -fold lower than the Ca^{2+} concentration in serum. This observation suggests that not all Ca^{2+} needs to be sequestered to restore *cnb1/cnb1* mutant survival in serum and that the MIC for growth inhibition by Ca^{2+} may be in the range of 2.5 to 3 mM. In addition, other calcium chelators may contribute to sequestering Ca^{2+} under these conditions. Interestingly, the addition of as little as 3 mM Ca^{2+} to BAPTA-chelated serum was sufficient to restore robust serum killing activity against calcineurin mutant cells, and lower concentrations of Ca^{2+} (1 or 2 mM) also restored toxicity, although in these cases there was higher variability in replicate assays (Fig. 4C). These findings support the conclusion that calcineurin is necessary to mediate *C. albicans* survival in response to stress induced by the levels of Ca^{2+} normally present in serum.

Crz1 and Crz2 have no individual roles in serum sensitivity. In *S. cerevisiae*, the Crz1/Tcn1 transcription factor is responsible for a majority of the transcriptional response to calcineurin-dependent activation in response to cell stress (33, 47), and two Crz1 homologs, Crz1 and Crz2, have recently

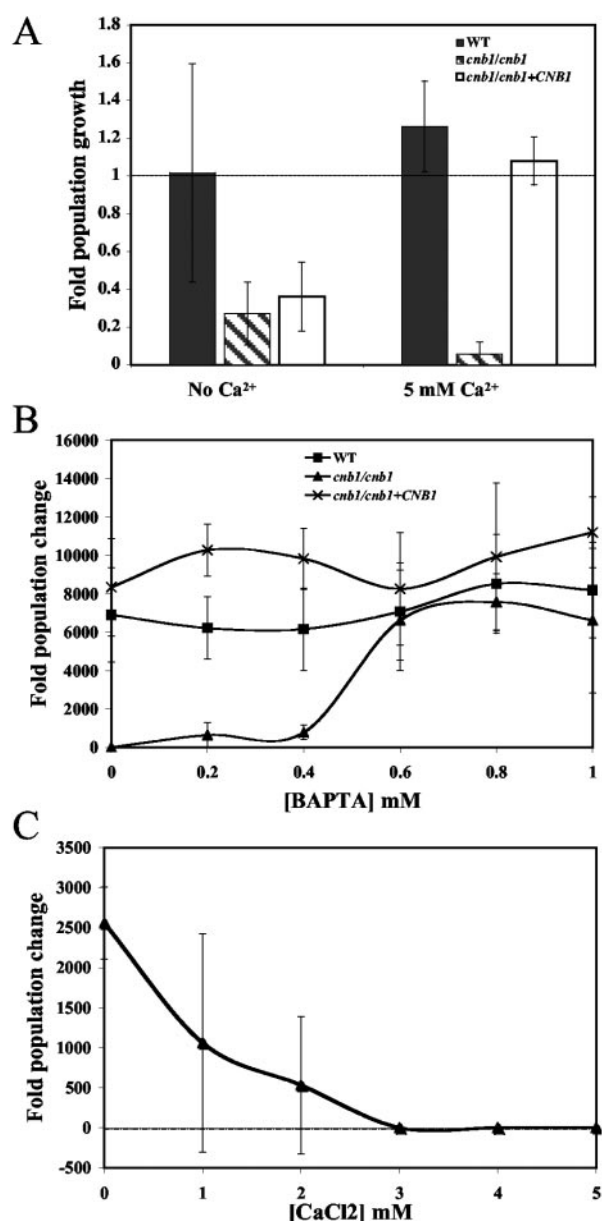


FIG. 4. Calcineurin is important for the response to stress induced by Ca^{2+} in serum. (A) The abilities of wild-type (SC5314) (solid bars), *cnb1/cnb1* (JRB64) (shaded bars), and *cnb1/cnb1+CNB1* (MCC85) (open bars) strains to persist in PBS were measured with or without 5 mM Ca^{2+} . (B) Wild-type (■), *cnb1/cnb1* (▲), and *cnb1/cnb1+CNB1* (×) strains were tested for their abilities to survive and proliferate in serum alone or in serum supplemented with the indicated concentrations of BAPTA. (C) The survival of the *cnb1/cnb1* calcineurin mutant strain was measured in serum with 0.8 mM BAPTA and supplemented with the indicated concentrations of Ca^{2+} . Cells were incubated at 30°C with (A) or without (B and C) agitation. The experiments for which results are shown in panels B and C were performed in 96-well plates.

been identified in *C. albicans* (5, 40). Crz1 plays a modest role in mediating azole resistance downstream of calcineurin, but from genetic analysis, it is clear that other downstream targets of calcineurin also participate in azole resistance (40). By contrast, Crz1 appears to play no role downstream of calcineurin

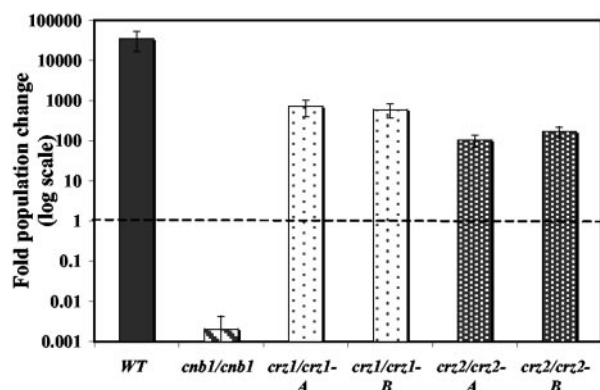


FIG. 5. Crz1 and Crz2 are not required for serum survival. Wild-type (SC5314) (WT), *cnb1/cnb1* (JRB64), *crz1/crz1* (OCC1.1 [bar A] and OCC3.8 [bar B]), and *crz2/crz2*Δ (DAY686 [bar A] and DAY697 [bar B]) strains were tested for their abilities to survive and proliferate in FBS. Cells were incubated at 30°C for 24 h.

in responding to salt-induced stress (40), whereas Crz2 is regulated by Rim101 at a high pH (pH 8) (5). Because the *S. cerevisiae* homolog Crz1 plays a critical role in mediating calcineurin responses, we tested whether these proteins play a role in the sensitivity of *C. albicans* calcineurin mutants to serum.

The abilities of *crz1/crz1* and *crz2/crz2* mutant strains to survive in 100% FBS were compared to those of the wild-type strain and *cnb1/cnb1* mutant cells. As originally observed, the wild-type strain readily proliferates in serum, while this medium is toxic to *cnb1/cnb1* mutant cells. Both the *crz2/crz2* and *crz1/crz1* mutant strains survived and proliferated in serum, albeit not quite to the wild-type level (~9 population doublings for *crz1/crz1*, ~7 for *crz2/crz2*, and ~15 for the wild-type strain) (Fig. 5). Thus, these putative transcription factors are unlikely to independently play any significant role downstream of calcineurin in responses to serum, suggesting that other calcineurin targets will be important in mediating *C. albicans* responses to serum stress and virulence.

DISCUSSION

Calcineurin is essential for the survival of *C. albicans* in serum, and we have shown here that calcineurin protects *C. albicans* from stress induced by endogenous levels of Ca^{2+} in this medium. Our original hypothesis was that a protein or antimicrobial peptide might be responsible for the killing of calcineurin mutant strains by serum, but the heat stability, small size, and proteinase K resistance of the killing activity in serum excludes this model. The finding that calcium mediates the lethal effect of serum against calcineurin mutants of *C. albicans* is surprising, because calcineurin mutants of *S. cerevisiae* are actually more resistant than wild-type cells to calcium stress (13). It has been suggested that the sensitivity of wild-type *S. cerevisiae* cells to calcium is due to a toxic effect of constant calcineurin activation, which is lacking in calcineurin mutant cells. It appears that *C. albicans* lacks this toxic effect of sustained calcineurin activity, perhaps because prolonged calcineurin signaling is common in the natural environment of *C. albicans* to which it has adapted. The results of Bader et al. (2)

showing that calcineurin is important for *C. albicans* to survive calcium stress in YPD medium provided the first clue that Ca^{2+} levels in serum might contribute to serum lethality.

Interestingly, addition of Ca^{2+} to YPD at levels similar to those in serum or PBS had no effect on calcineurin mutant survival. YPD may contain natural calcium chelators that reduce the effective Ca^{2+} concentration in the medium, or the differing metabolic rates of cells in a rich growth medium versus serum may alter physiological responses to Ca^{2+} ions. Alternatively, additional components in serum may contribute to the lethal effect on calcineurin mutant strains in conjunction with Ca^{2+} . Our assays were designed to detect the major component of serum contributing to its toxic activity against the calcineurin mutants, and minor contributing components may have thus far escaped detection. Any additional toxic serum component(s), however, remains hypothetical at present.

Why is calcineurin required to survive stress caused by Ca^{2+} in serum? Based on the finding that mutants deficient in Crz1 or Crz2 (homologs of the transcription factor Crz1/Tcn1, which is responsible for the transcriptional response downstream of calcineurin in *S. cerevisiae*) are not as sensitive to serum as calcineurin mutant cells, this response may not be transcriptional. Isolation and analysis of a *crz1/crz1 crz2/crz2* double-mutant strain will be required to exclude models in which the two might play a redundant role in serum survival. An alternative model is that the role of calcineurin in serum survival may be mediated by posttranslational regulation of downstream targets. Attractive candidate calcineurin targets in this setting include homologs of *S. cerevisiae* Hph1, recently reported as a direct calcineurin target (22), and Ca^{2+} pumps on the cell surface and in intracellular organelles (such as Cch1-Mid1 on the plasma membrane, Pmc1 in the vacuole, and Pmr1 in the Golgi apparatus), which are known to be controlled by calcineurin in *S. cerevisiae* (14). The integral membrane protein Hph1 is an attractive downstream candidate because, in combination with its close homolog Hph2, it is essential for cell survival of stress induced by high NaCl concentrations, alkaline pHs, and cell wall perturbation (22). However, exhaustive database searches of the *C. albicans* genomic sequencing project at Stanford University reveal no clear Hph1 or Hph2 homolog in *C. albicans* (J. Reedy, J. R. Blankenship, C. Onyewu, and J. Heitman, unpublished results). The ionic pumps Cch1-Mid1, Pmc1, and Pmr1 are attractive downstream targets because they modulate the Ca^{2+} concentration in the cytoplasm and in organelles where calcium can play an essential role as a cofactor (in the endoplasmic reticulum, for example, as a cofactor for protein chaperones). Misregulation of these pumps could lead to an accumulation of toxic levels of Ca^{2+} in the cytoplasm, leading to aberrant calcium signaling, perhaps through calmodulin, or to a loss of calcium in organelles that require Ca^{2+} for proper functioning. These possibilities are currently being investigated.

The natural niche of *C. albicans* is the mucosal surfaces of the gastrointestinal tract of mammals. *C. albicans* typically lives as a commensal within its host, and until the introduction of immunosuppressive therapy in the last century, serious systemic infections were uncommon (37). With the advent of immunosuppressive therapy for cancer chemotherapy and as antirejection therapy for organ transplant recipients, systemic

Candida infections have become increasingly common. To make the transition from a commensal organism to a systemic pathogen, *C. albicans* must first enter the bloodstream. It can do so by taking advantage of medical devices, such as intravenous catheters, to enter the bloodstream directly (reviewed in reference 29), or it can cross intact or damaged gastrointestinal mucosae into the bloodstream (9). The bloodstream is a hostile environment, and not only does *C. albicans* face a radical change in nutrient availability; it is also exposed to innate immune defenses, including plasma, macrophages, and neutrophils. Although free-flowing *C. albicans* appears to be rapidly cleared from the blood (3, 42), the fungus does persist in the bloodstream (~10% in an experimental rabbit infection model) (3). This persistent bloodstream infection likely contributes to dissemination, but whether candidemia has any additional role in disease progression besides acting as a vehicle for rapid dissemination is not known. It is also unclear how long *C. albicans* remains exposed to the bloodstream when attached to the endothelium of blood vessels before it can successfully invade to colonize host tissues and organs. Calcineurin is part of the arsenal that *C. albicans* deploys to survive the hostile bloodstream environment in addition to germ tube formation, host cell adherence, and host cell injury.

Although the Ca^{2+} -sensitive phenotype of calcineurin mutant strains of *C. albicans* in serum derived from whole blood is described here, it is likely that calcineurin is also important for mediating survival of the fungus in the interstitial fluids present in organs such as the kidney, where free Ca^{2+} concentrations can be quite high. In the standard American adult diet, approximately 1 g of calcium is ingested daily, and ~200 mg of this Ca^{2+} is renally excreted (8). The concentration of Ca^{2+} in the kidney is highest in the nephrons, but surrounding cells may be exposed to elevated levels as well, and calcineurin might enable *C. albicans* to survive these conditions. Indeed, *C. albicans* calcineurin mutants colonize the kidney poorly (7), and this could be due in part to the Ca^{2+} levels present in the interstitial fluids of this organ.

The risk of death in immunocompromised patients diagnosed with disseminated candidiasis is high, even with current therapy. Although treatment options have increased with the recent introduction of voriconazole and caspofungin, the armamentarium of antifungal drugs to combat candidiasis remains limited. Calcineurin is an attractive drug target: it is essential for *C. albicans* virulence, and drug treatment should halt dissemination of the infection. Furthermore, calcineurin-targeting drugs already exist, and the crystal structure of the interaction of the drugs with calcineurin has been solved (19, 24, 26, 27), potentially enabling rational design of analogs that will impair fungal calcineurin-dependent virulence but spare calcineurin-dependent signaling events critical for immune responses in the host. Furthermore, several studies have documented a potent fungicidal synergism between calcineurin-inhibiting drugs and drugs that inhibit the ergosterol biosynthetic pathway (6, 11, 31, 32, 39), making calcineurin an even more attractive antifungal-drug target.

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